Emergence of NK1.1$^+$ Cells as Effectors of IFN-γ Dependent Immunity to *Toxoplasma gondii* in MHC Class I-deficient Mice

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Summary

CD8$^+$ T lymphocytes have been reported to play a major role in the protective immune response against acute infection with *Toxoplasma gondii*. In order to further assess the role of CD8$^+$ cells in resistance against this protozoan we examined the ability of β_2m-deficient mice, which fail to express MHC class I molecules and peripheral CD8$^+$ lymphocytes, to survive tachyzoite challenge following vaccination with an attenuated parasite mutant. Surprisingly, vaccination of β_2m-deficient mice induced strong resistance to lethal challenge, with >50% surviving beyond 3 months. Vaccinated β_2m-deficient mice, but not control heterozygotes, showed a five- to six-fold expansion in spleen cell number and ~40% of the splenocytes were found to express the NK markers NK1.1 and asialo GM_1. Spleen cells from the vaccinated β_2m-deficient animals failed to kill either infected host cells or the NK target YAC-1. However, high levels of IFN-γ were secreted when the cells were cultured in vitro with soluble *T. gondii* lysate, and this response was abolished by NK1.1$^+$ but not CD4$^+$ and CD8$^+$ lymphocyte depletion, implicating the NK1.1$^+$ population as the major source of IFN-γ. More importantly, vaccine-induced immunity in β_2m-deficient mice was completely abrogated by in vivo administration of antibody to NK1.1, asialo GM_1, or IFN-γ. Together, the data suggest that in class I–deficient mice vaccinated against *T. gondii*, the absence of CD8$^+$ effector cells is compensated for by the emergence of a population of NK1.1$^+$ and asialo GM_1^+ cells which lack cytolytic activity, and that the protective action of these cells against the parasite is attributable to IFN-γ production. The induction of this novel NK population may provide an approach for controlling opportunistic infections in immunocompromised hosts.

Infection with the intracellular protozoan *Toxoplasma gondii* is characterized by an acute proliferative stage, during which infective tachyzoites invade and replicate within a wide variety of host cells, and a chronic slow growing phase consisting of parasite encystment within tissues of the brain and muscle. Although infection is usually innocuous, in immunocompromised hosts encysted parasites can reactivate, leading to uncontrolled tachyzoite proliferation, tissue damage, and encephalitis, which in some cases leads to host death (1).

Control of both acute and chronic infection is largely dependent upon the cytokine IFN-γ. Thus, neutralization of endogenous IFN-γ by in vivo administration of mAb renders mice susceptible to primary infection with the normally avirulent *T. gondii* strain ME49 (2) and ablates protective immunity induced by vaccination with the attenuated mutant ts-4 (3). Prevention of toxoplasma encephalitis is also dependent upon the activity of IFN-γ, since treatment of chronically infected mice with anti–IFN-γ mAb leads to reactivation of acute infection (4, 5), and administration of recombinant IFN-γ reduces both tachyzoite numbers and inflammation in these animals (6).

While both CD4$^+$ and CD8$^+$ T lymphocytes from immune mice secrete large amounts of IFN-γ in vitro in response to tachyzoites (7), CD8$^+$ cells are likely to be the major source of T cell–derived IFN-γ in vivo. Thus, immune CD8$^+$ cells transfer resistance to naive recipients more efficiently than CD4$^+$ cells (8), and this effect is abrogated by simultaneous treatment with anti-IFN-γ mAb (9). Similarly, vaccine-induced immunity is abrogated by CD8$^+$ cell depletion, and while CD4$^+$ depletion during vaccination results in failure to generate protection, it has no effect on resistance to challenge infection once immunity is established (3). On the basis of this evidence, it has been argued that CD8$^+$ cells are the major effectors of the IFN-γ–dependent protective response and that their generation is dependent upon CD4$^+$ helper function (7, 8).

In order to further define the role of $CD8^+$ T lymphocytes in immunity to Toxoplasma, we examined the response of MHC class I-deficient mice following vaccination with the attenuated *T. gondii* mutant ts-4. These animals were constructed by targeted disruption of the gene encoding the β_2 microglobulin (β_2m) subunit of the class I molecule (10). As a result, the peripheral $CD8^+$ compartment fails to develop (11) and the mice fail to display normal resistance to infection with *Trypanosoma cruzi* (12), *Mycobacterium tuberculosis* (13) and certain viruses (14). As described below, β_2m-deficient mice unexpectedly were found to develop high levels of protective immunity to *T. gondii* following vaccination. This resistance was associated with a massive parasite-induced expansion of a splenocyte population expressing NK1.1 and asialo GM_1 ($ASGM_1$),[1] phenotypic markers characteristic of NK cells. The latter population, while producing IFN-γ in response to parasite Ag, was unable to mediate lysis of either *T. gondii*–infected host cells or conventional NK targets, and thus appears to be functionally unique. The appearance of these cells in $CD8^+$ deficient animals suggests that they could be induced as alternative effectors of parasite immunity in immunodeficient hosts.

Materials and Methods

Mice and Parasites. Chimeric mice homozygous (−/−) for the disrupted β_2m gene were derived from (129 × B6)F2 founder stock as described (10). Animals used in this study were from the fifth backcross generation. Control animals heterozygous for the inactive β_2m gene (+/−) were obtained by crossing −/− mice with C57Bl/6 animals purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were bred and maintained under specific pathogen-free conditions. The RH and ts-4 strains of *T. gondii* were maintained by weekly passage in vitro on human fibroblasts at 37°C and 34°C, respectively.

T. gondii Ag Preparation. Soluble *T. gondii* Ag was prepared as described in detail elsewhere (15). Briefly, RH tachyzoites harvested from the peritoneal cavity of Swiss-Webster mice were sonicated in the presence of protease inhibitors, centrifuged at 10,000 *g*, dialyzed into PBS, filtered through a 0.2-μm membrane (Costar Corp., Cambridge, MA), and stored in aliquots at −80°C until use.

Antibodies. The following directly conjugated mAbs (obtained from PharMingen, San Diego, CA) were used for flow cytometric analyses: FITC labeled AF6-88.5 (anti-H-$2K^b$), FITC labeled AMS-32.1 (anti-IA^d), FITC labeled 145-2C11 (anti-CD3-ϵ), PE labeled 500A2 (anti-CD3-ϵ), FITC labeled RM4-5 (anti-CD4), PE labeled RM4-5, FITC labeled 53-5.8 (anti-CD8), PE labeled H57-597 (anti-$\alpha\beta$ TCR), PE labeled GL3 (anti-$\gamma\delta$ TCR), PE labeled 30-H12 (anti-Thy1.2), FITC labeled PK136 (anti-NK1.1) (16), and PE labeled 5E6 (reactive with a subpopulation of NK cells) (17). Biotinylated mAb 30F11.1 (anti-CD45) and 145-2C11 were purchased from PharMingen. Anti-$ASGM_1$ mAb (18) was kindly provided by Dr. T. Higgins (University of Pennsylvania) and biotin labeled as described (19). The antibodies were used at dilutions predetermined to give optimal staining by flow cytometric analysis.

For in vivo cell depletions mAb XMG1.2 (anti-IFN-γ), PK136, GK1.5 (anti-CD4), and GL113 (anti-β-galactosidase), rabbit antiserum specific for $ASGM_1$ (Wako Chemicals, Richmond, VA), and normal rabbit serum were used. The Ab were partially purified by precipitation from ascites fluid with 40% ammonium sulfate, and sterilized by filtration through a 0.2-μm membrane (Costar Corp.) prior to use. In vitro depletions employed hybridoma culture supernatants of mAb RL172.4 (anti-CD4), 3-155 (anti-CD8), and SW3A4 (anti-NK1.1).

Vaccination and Challenge. Mice were vaccinated by biweekly intraperitoneal injections of 2×10^4, 2×10^5, and 2×10^5 ts-4 tachyzoites, then challenged 2 wk later by subcutaneous injection of 2000 virulent RH strain tachyzoites.

In Vivo Cell Depletion. To assess the role of NK cells and IFN-γ in resistance, some groups of mice were depleted of $NK1.1^+$ cells or IFN-γ by twice weekly injections of 2 mg of mAb PK136 (16) or XMG 1.2 i.p. beginning 3 d prior to challenge. Control mAb (GL113) was administered under similar conditions. $ASGM_1^+$ cells were depleted by twice weekly injection of 50 μl i.p. of rabbit antiserum specific for $ASGM_1$. Similarly prepared normal rabbit serum was administered to mice under identical conditions. Efficacy of cell depletions, determined by flow cytometric analysis, was 90–95%.

In Vitro Cell Depletion. Splenocytes were depleted of specific cell populations using mAb and rabbit serum as a source of complement (Accurate Chemical and Scientific Corp., Westbury, NY). Cells (1.5×10^8) were incubated in 2.5 ml mAb-containing culture supernatant (45 min, 0°C), washed, incubated (45 min, 37°C) in 5 ml of a 1:10 dilution of rabbit serum as a source of complement (Accurate Chemical and Scientific Corp.), washed, and the Ab + complement treatment was repeated. Specificity of depletions was confirmed by flow cytometry.

Flow Cytometric Analysis. Spleen cells were first treated with ammonium chloride/potasium bicarbonate lysis buffer (B and B Research Laboratories, Inc., Fiskeville, RI) to remove red cells. Samples containing 10^6 cells were incubated with Ab (diluted in 50 μl HBSS supplemented with 1% FCS and 0.1% sodium azide) for 30 min at 0°C, and then washed. Staining was performed in the presence of saturating levels of unlabeled Fc receptor specific antibody (2.4G2) to block nonspecific Fc receptor binding. In the case of biotin-labeled mAb, after Ab staining cells were washed and incubated (30 min, 0°C) in avidin conjugated to PE (PharMingen; 50 μl of a dilution predetermined to be optimal). Cells were washed and analyzed on an EPICS 753 flow cytometer (Coulter Corporation, Hialeah, FL) for 1- and 2-color analyses (10,000 cells per sample). Dead cells were excluded by propidium iodide gating.

Measurement of In Vitro IFN-γ Response. Splenocytes and splenocyte subpopulations from ts-4 vaccinated mice (5×10^6/well or 3×10^5/well, as indicated) were cultured for 72 h in the presence of soluble *T. gondii* Ag (50–100 μg/ml) and IFN-γ was measured in a two-site capture ELISA using immobilized mAb HB170 (anti–IFN-γ), rabbit polyclonal anti–mouse IFN-γ, and peroxidase-conjugated donkey anti–rabbit Ig (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Cytolytic Assays. Splenocytes from nonvaccinated, ts-4 vaccinated, and poly I:C injected mice were tested for the ability to kill the NK sensitive target YAC-1 in a ^{51}Cr release assay. To induce NK cell activity, 50 μg poly I:C (Sigma Chemical Co., St. Louis, MO) was administered intraperitoneally to nonvaccinated mice 24 h before assay. ^{51}Cr-labeled YAC-1 targets (10^4/well) were incubated with splenocytes, and chromium released into the supernatant was measured after 5 h using a supernatant harvesting press (Skatron, Lier, Norway).

Lytic activity of splenocytes for *T. gondii*–infected target bone marrow macrophages was measured in a ^{51}Cr release assay as previously described (20). Briefly, target macrophages were cultured

[1] *Abbreviation used in this paper:* $ASGM_1$, asialo GM_1.

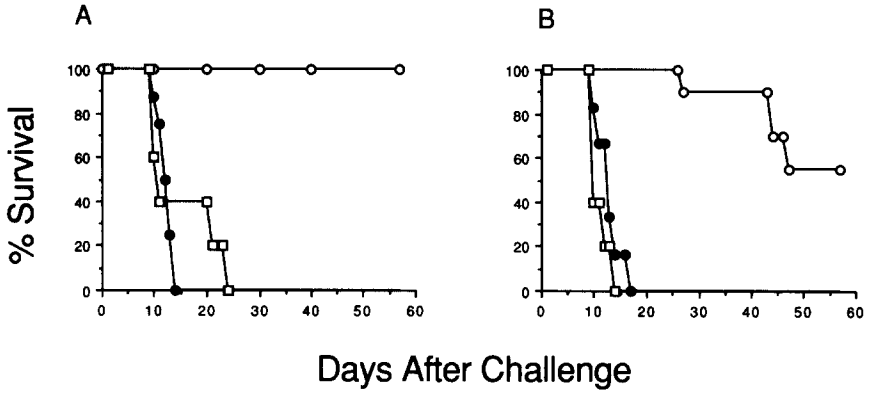

Figure 1. Survival of vaccinated β_2m-deficient (−/−) and nondeficient (+/−) mice following *T. gondii* challenge. *A*, +/− mice; *B*, −/− mice. Closed circles, nonvaccinated animals; open circles, ts-4 vaccinated; open squares, ts-4 vaccinated and treated with anti-IFN-γ mAb. See Materials and Methods for details. 5–8 animals were used per group. Essentially identical results were obtained in three independent experiments.

for 5–7 d in L cell media, infected with ts-4, and then 18 h later washed, labeled with ^{51}Cr, and incubated with effectors for 5 h. Effector splenocytes were obtained from vaccinated mice and used immediately, or cultured for 7 d with ts-4 (7.5:1 ratio of spleen cells to tachyzoites) before assay. For both types of cytolytic assays the results are expressed as the mean ± standard deviation of the percent release values from individual mice (three to four per group).

Results

β_2m-deficient Mice Develop High Levels of Resistance to T. gondii challenge. Both nondeficient +/− and deficient −/− mice (heterozygous and homozygous, respectively, for the defective β_2m gene) succumbed within 14 d when inoculated with the virulent *T. gondii* strain RH without prior vaccination (Fig. 1). As previously shown for other mouse strains (3, 8, 21), +/− animals when vaccinated with tachyzoites of the temperature-sensitive mutant ts-4 showed complete resistance to challenge with RH (Fig. 1 *A*). Unexpectedly, β_2m-deficient −/− mice also displayed a high degree of resistance following *T. gondii* vaccination, with 55% of the animals surviving for at least 60 d after challenge (Fig. 1 *B*). Resistance in both mouse strains was dependent upon IFN-γ, since treatment of mice with antibody to this cytokine completely abrogated immunity (Fig. 1, *A* and *B*). Administration of a rat IgG mAb of irrelevant specificity (β-galactosidase) had no effect on the ability of either stain to resist challenge infection (data not shown).

Vaccination of β_2m-deficient Mice Induces Expansion of NK1.1$^+$ Cells. In order to investigate the mechanism by which β_2m-deficient mice resist RH challenge, we examined the splenocyte populations from vaccinated and nonvaccinated animals. A dramatic five- to six-fold increase in spleen cell number was observed in ts-4 immunized −/− animals. In contrast, no significant increase in splenocytes was seen in vaccinated +/− or nonvaccinated −/− mice. As expected, flow cytometric analysis revealed only low levels of class I (H-2K^b) and CD8 expression in spleen cells from −/− animals (Table 1). In addition, no CD8$^+$ lymphocytes were detected in the peritoneum (data not shown), a site where these cells have recently been detected in β_2m-negative mice injected with tumor cells (22). Instead, we found a striking increase (from 5 to 39%; Table 1 and Fig. 2) in the percent of splenocytes expressing the NK marker NK1.1 in vaccinated −/− animals. In contrast, only a minor increase in NK1.1$^+$ cells (3–7%) was induced by vaccination of +/− animals. The level of CD4$^+$ lymphocytes was 10–15% lower in vaccinated β_2m-deficient than in nonvaccinated −/− and vaccinated +/− mice, this decrease most probably reflecting the increased percent of NK1.1$^+$ cells.

Fig. 2 *D* shows the population of anti-NK1.1 staining spleen cells in vaccinated β_2m-negative mice selected for analysis of other surface markers by dual fluorescence. Since there was overlap between positive and negative staining cells, only the brightest 20% of total splenocytes was examined in order

Table 1. *Spleen Cell Composition in Vaccinated and Nonvaccinated −/− and +/− Mice**

	% Positive			
	H-2K^b	CD4	CD8	NK1.1
−/− vaccinated	6.5 ± 1.5†	14.1 ± 3.0	1.8 ± 0.9	39.4 ± 9.3
+/− vaccinated	98.2 ± 1.0	24.3 ± 1.1	16.2 ± 0.9	7.3 ± 1.6
−/− nonvaccinated	2.0 ± 0.1	29.5 ± 2.3	1.5 ± 0.8	5.1 ± 1.0
+/− nonvaccinated	96.3 ± 2.1	16.1 ± 3.1	8.4 ± 1.1	3.3 ± 0.7

* Splenocytes were stained with FITC conjugated mAb specific for the indicated markers. See Materials and Methods for details.
† Mean ± SD of individual mice (3–7 per group). Similar results were obtained in 5 independent experiments.

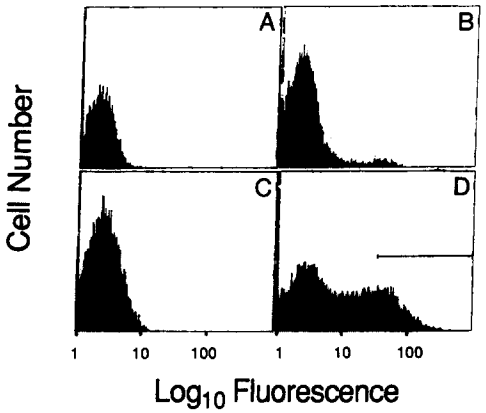

Figure 2. NK1.1 expression by vaccinated +/− and −/− mice. Splenocytes from +/− mice (*A* and *B*) and −/− (*C* and *D*) animals were stained with a fluoresceinated irrelevant mouse IgG (anti-IA^d) (*A* and *C*) or anti-NK1.1 (*B* and *D*).

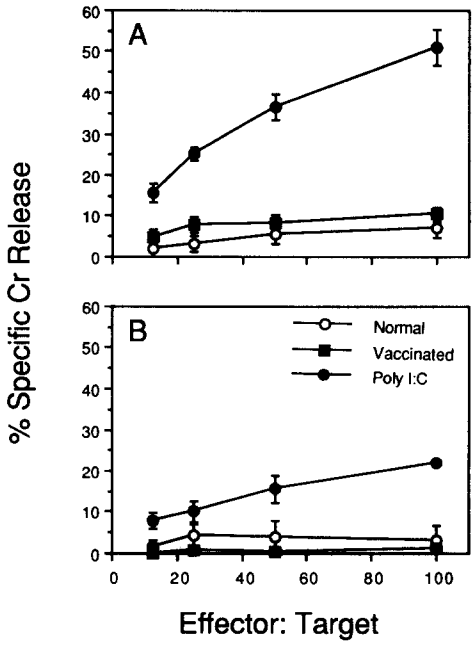

Figure 4. NK cells induced by ts-4 vaccination of β_2m-deficient mice have defective cytolytic activity against YAC-1 targets. Splenocytes from nonvaccinated, ts-4 vaccinated, and poly I:C injected +/− (*A*) and −/− (*B*) mice were tested for the ability to kill the NK sensitive target YAC-1. The results show mean ± SEM (four mice per group) and are representative of those obtained in four additional experiments.

to ensure exclusion of NK1.1$^-$ cells. In this cell staining experiment, as in the others shown, saturating levels of unlabeled mAb 2.4G2 were included to block nonspecific Fc receptor binding. Cells stained with FITC-labeled NK1.1$^+$ alone showed no PE fluorescence (Fig. 3 *A*) but virtually all of the NK1.1$^+$ cells expressed ASGM$_1$ (Fig. 3 *B*). In contrast, minimal staining was detected with mAb specific for Thy1.2 (5%; Fig. 3 *C*), CD4 (2%; Fig. 3 *D*), and $\alpha\beta$ TCR (3%; Fig. 3 *F*). However, a small amount of staining (12%; Fig. 3 *H*) was detected using 5E6, a mAb detecting a marker associated with a subpopulation of NK cells (17). In addition, a minor population of NK1.1$^+$ cells was found to express CD3 (14%; Fig. 3 *E*) and $\gamma\delta$ TCR (14%; Fig. 3 *G*). The similar proportion of NK1.1$^+$ cells expressing the latter markers suggested they were produced by the same subpopulation of cells, and indeed 3-color cytometric analysis using mAb to NK1.1, CD3, and $\gamma\delta$ TCR confirmed this to be the case (data not shown).

Cytotoxic Activity of T. gondii-induced NK1.1$^+$ Cells. The large increase in NK1.1$^+$ cells was unexpected because it has recently been shown that β_2m-deficient mice are defective in NK cell lytic activity (23, 24). We similarly found that poly I:C treatment of nonvaccinated −/− mice resulted in a much smaller enhancement of cytolytic function relative to that stimulated in +/− mice (Fig. 4). Moreover, splenocytes from *T. gondii* vaccinated class I-deficient mice, although composed of up to 40% NK1.1$^+$ cells, were unable to lyse YAC-1 targets (Fig. 4 *B*).

The ability of splenocytes from vaccinated mice to kill tachyzoite-infected host cells was also assessed. As shown previously (20, 25), vaccination of normal (+/−) animals generated splenic effector cells capable of lysing parasite-infected bone marrow macrophages (Fig. 5 *A*). A low but significant level of killing (10% specific ^{51}Cr-release; $p < 0.05$) was detected using restimulated +/− effectors and infected −/− targets (Fig. 5 *A*). The latter could possibly be mediated by a low level of functionally conformed cell surface class I heavy

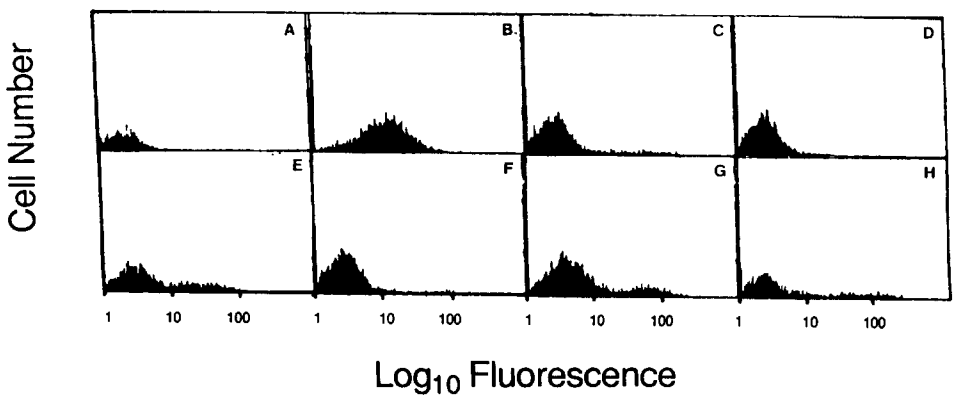

Figure 3. Surface markers associated with parasite-induced NK1.1$^+$ cells in β_2m-deficient mice. Spleen cells from vaccinated −/− mice were double stained with FITC conjugated anti-NK1.1 and PE labeled mAb. The results show PE fluorescence displayed by NK1.1$^+$ cells stained double stained with: *A*, nothing; *B*, PE-anti-ASGM$_1$; *C*, PE-anti-Thy1.2; *D*, PE-anti-CD4; *E*, PE-anti-CD3; *F*, PE-anti-$\alpha\beta$ TCR; *G*, PE-anti-$\gamma\delta$ TCR; *H*, PE-anti-5E6. These results show an analysis from three pooled spleens. Essentially identical results were obtained in three independent experiments.

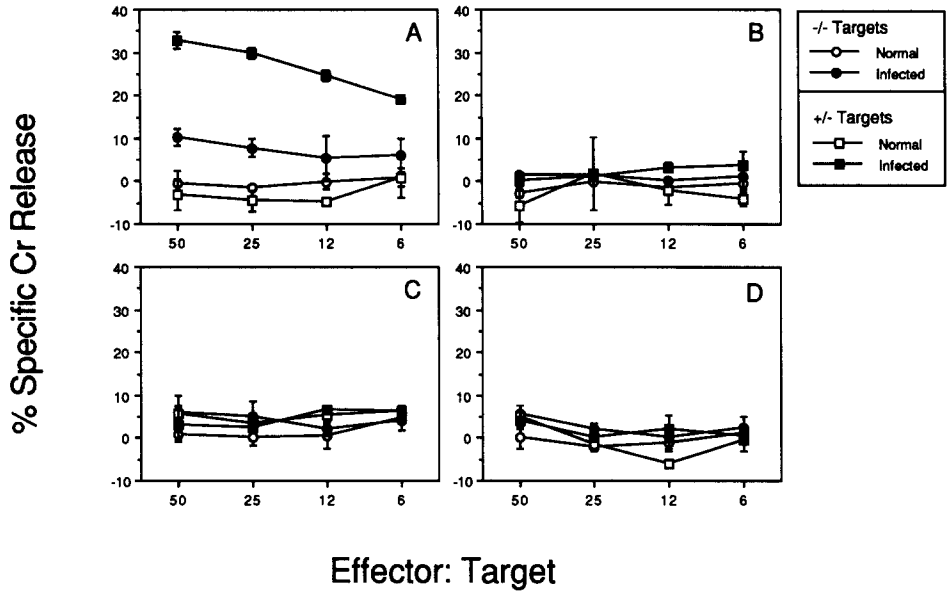

Figure 5. Cytolytic activity of splenocyte effectors for *T. gondii*-infected cells. Spleen cells from ts-4 vaccinated mice were either cultured for 7 d with ts-4 prior to assay (*A* and *B*), or used immediately in the CTL assay (*C* and *D*). *A* and *C* show CTL activity of +/− effectors; *B* and *D* show CTL activity of −/− effector cells. Bone marrow derived macrophage target cells were either not infected or infected with ts-4 as indicated prior to ^{51}Cr-labeling. The results show mean ± SEM (3–4 mice per group). Similar results were obtained in three independent experiments.

chain expressed by β_2m-deficient animals (26), or alternatively by exogenous β_2m supplied by the serum in the culture medium. In contrast, neither ts-4 restimulated (Fig. 5 *B*) nor freshly isolated splenocytes (Fig. 5 *D*) from −/− animals lysed to a significant degree infected targets of either strain. Taken together, the results in Figs. 5 and 6 show that the parasite-induced NK1.1$^+$ cells in β_2m-deficient mice have no detectable lytic activity against either *T. gondii*-infected targets or the classic NK target YAC-1.

NK1.1$^+$ Cells Induced in β_2m-deficient Mice Produce IFN-γ in Response to T. gondii Ag. NK cells are a major source of IFN-γ (27) and *T. gondii* has recently been shown to trigger in vitro production of this cytokine by splenic NK cells from SCID mice (28). Therefore, we assessed the ability of vaccine-induced NK1.1$^+$ cells to produce IFN-γ in response to the parasite. Nondepleted splenocytes from β_2m-deficient and nondeficient animals when stimulated with soluble *T. gondii* Ag produced 30 and 53 ng/ml of the cytokine, respectively (Table 2), while unstimulated cells synthesized less than 2 ng/ml (data not shown). Depletion of CD4$^+$ and CD8$^+$ lymphocytes, but not NK1.1$^+$ cells, from vaccinated nondeficient animals eliminated the parasite-induced IFN-γ response. In contrast, in vaccinated class I-deficient mice depletion of NK1.1$^+$ cells, but not T cells, abolished *T. gondii*–stimulated IFN-γ production (Table 2). Similarly, after in vivo NK cell depletion, splenocytes synthesized only low levels of IFN-γ (7 ng/ml) following in vitro stimulation with parasite extract, and this response was abolished by lysis of T cells prior to culture (Table 2). In addition, removal of CD3$^+$ $\gamma\delta$TCR$^+$ cells in the NK1.1$^+$ population by cell sorting did not alter the ability of the remaining cells to secrete IFN-γ in response to parasite Ag (data not shown).

Vaccine-induced Protection in β_2m-deficient Mice is Dependent Upon NK1.1$^+$ and ASGM$_1^+$ Cells. Since the vaccine-induced NK1.1$^+$ cells produce IFN-γ in vitro in response to *T. gondii* triggering (Table 2), and the protective response in class I-deficient animals is dependent upon this cytokine (Fig. 1 *B*), we assessed the effects of in vivo depletion of NK cells on the resistance of these mice to challenge infection. While administration of anti-NK1.1 mAb or rabbit anti-

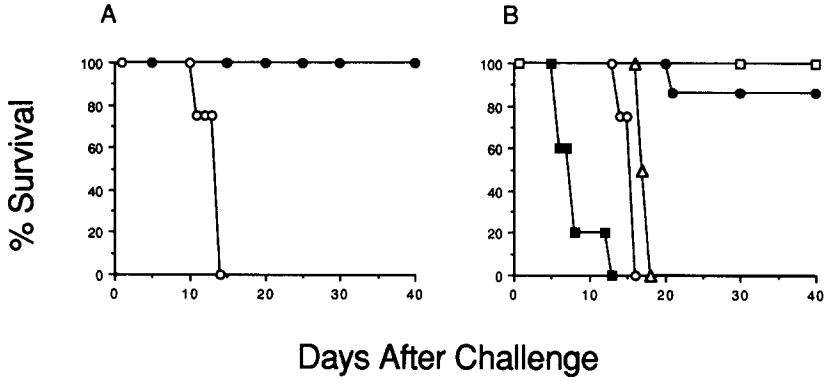

Figure 6. Treatment of vaccinated β_2m-deficient mice with anti-NK antibodies abrogates protective immunity to challenge infection. *A*, +/− mice; *B*, −/− mice. In *A*, animals were either not vaccinated (*open circles*), vaccinated, vaccinated and treated with anti-NK1.1 mAb, or vaccinated and treated with anti-ASGM$_1$ antibodies. Since none of the mice in the latter 3 groups succumbed to challenge they are represented with one line (*closed circles*). In panel B, animals were either not vaccinated (*open circles*), vaccinated (*closed circles*), vaccinated and treated with anti-NK1.1 mAb (*open triangles*), vaccinated and treated with anti-ASGM-1 (*closed squares*), or vaccinated and treated with normal rabbit serum (*open squares*). 4–6 mice were used per group. The results shown are representative of those observed in 2 experiments.

Table 2. *Source of IFN-γ in T. gondii Ag-stimulated Splenocytes From Vaccinated Mice**

Mouse strain	Treatment	IFN-γ
		ng/ml
+/−	Nondepeleted	48.4 ± 2.1
	T cell depleted	1.7 ± 0.6
	NK cell depleted	47.2 ± 2.0
−/−	Nondepleted	30.2 ± 3.2
	T cell depeleted	22.1 ± 2.3
	NK cell depleted	2.3 ± 0.1
−/−	NK cell depleted in vivo	6.7 ± 0.2
	NK cell depleted in vivo, T cell depleted in vitro	0.8 ± 0.6

* Indicated cell populations were treated with anti-CD4 + anti-CD8 mAb (T cell depletion) and anti-NK1.1 mAb (NK cell depletion) + rabbit complement and then 5 × 10^6 cells were cultured in vitro with 100 μg/ml soluble *T. gondii* Ag for 72 h before measuring IFN-γ. See Materials and Methods for details. This experiment is representative of 3 performed.

$ASGM_1$ antibodies failed to alter the ability of vaccinated +/− mice to resist challenge infection (Fig. 6 *A*), the same antibody treatments were found to completely abrogate vaccine-induced immunity in β_2m-deficient animals (Fig. 6 *B*). As expected, $CD8^+$ depletion did not alter the ability of the class I-deficient mice to resist challenge (data not shown).

Discussion

Immunity to *T. gondii* is dependent upon IFN-γ and, in immunologically intact mice, $CD8^+$ lymphocytes appear to be major producers of this cytokine. The results presented in this paper demonstrate that in the absence of $CD8^+$ cells, a previously uncharacterized population of effector cells emerge in response to *T. gondii* vaccination. These cells express NK1.1 and $ASGM_1$, markers associated with NK cells, and their elimination by in vivo anti-NK1.1 or anti-$ASGM_1$ Ab treatment ablates immunity to challenge infection. The parasite-induced $NK1.1^+$ cells, while failing to lyse either the NK target YAC-1 or *T. gondii*-infected bone marrow macrophages, release high levels of IFN-γ when cultured with tachyzoite Ag. The production of this cytokine by the $NK1.1^+$ population is likely to account for their protective effect since depletion of IFN-γ in vivo abrogates immunity in the $CD8^+$-deficient mice.

Our finding that vaccinated class I-deficient mice resist *T. gondii* challenge contrasts with previously reported experiments demonstrating an inability of these animals to survive infection with *Trypanosoma cruzi* (12), *Mycobacterium tuberculosis* (13), and a virulent influenza strain (14). However, β_2m-negative mice are able to clear infections with other influenza strains (14, 29) as well as vaccinia and Sendai virus (30, 31). Indeed, while not as striking as the response to *T. gondii*, vaccinia-infected β_2m-negative mice have greater numbers of spleen cells than do normal heterozygotes, and part of this increase is due to non-B, non-T cells (30). Therefore, it is possible that control of these infections is attributable to the induction of a protective $NK1.1^+$ cell population similar to that described here.

Recent reports have suggested that class I molecules play a role in driving differentiation of NK cells, as evidenced by decreased cytolytic activity of poly I:C induced NK cells from β_2m-deficient mice (23, 24). We also found that poly I:C treatment of nonvaccinated −/− mice resulted in a much smaller enhancement of cytolytic function relative to that stimulated in +/− mice. Furthermore, $NK1.1^+$ cells from vaccinated mice fail to exhibit cytolytic activity, although they secrete IFN-γ in response to parasite Ag. These observations demonstrate that target cell killing and cytokine (IFN-γ) production are independent and dissociable activities of $NK1.1^+$ and $ASGM_1^+$ cells, and that intact class I dimers per se, while important for generation of cytolytic NK activity (32), do not appear to be required for differentiation of cytokine-secreting cells of this phenotype. We do not know at present if the parasite-induced $NK1.1^+$ cells are derived from an independent lineage relative to cytolytic NK cells, or whether the cytolytic mechanism of these cells is inoperational by virtue of the fact that they are generated in the absence of class I molecules.

The results presented here suggest that in the immune system of class I-deficient animals loss of IFN-γ–producing $CD8^+$ lymphocytes is compensated for by production of an unconventional effector cell population capable of producing the same cytokine. The production of novel effector cells in response to immunodeficiency has also been observed in β_2m-negative mice infected with Sendai virus (31). Such mice clear the virus, albeit with delayed kinetics relative to normal mice, and although $CD8^+$ CTL activity is responsible for virus elimination in class I-expressing animals, in the immunodeficient mice clearance is attributable to the appearance of $CD4^+$ CTL. Similarly, infection of β_2m-negative animals with murine lymphocytic choriomeningitis virus induces splenic $CD4^+$ CTL activity (33). However, it is unlikely that $CD4^+$ cytolysis plays a major role in the protective response of class I-deficient mice infected with *T. gondii* because virtually no $CD4^+$ cells were detected in the protective $NK1.1^+$ population, and no cytolytic activity was detected against parasite-infected cells using splenocyte effectors from vaccinated β_2m-negative mice. In addition, immunity was completely eliminated by IFN-γ depletion, implicating this cytokine as the major mediator of immunity rather than cell-mediated cytolytic activity. Nevertheless, since IL-2 induces NK cell proliferation (34), conventional $CD4^+$ lymphocytes could play a helper role in the induction and activity of these cells; this possibility is currently being examined.

Depletion of $NK1.1^+$ cells in ts-4 vaccinated +/− mice had no effect on resistance to *T. gondii*, indicating that such cells probably do not play a major role during the effector phase of immunity in these animals. However, NK cell lytic activity has been detected in other experimental models used to study *T. gondii* (35–37) and it is possible that in these cases,

as with the class I-deficient mice, NK1.1$^+$ cells provide a source of protective IFN-γ. In addition, parasite induction of NK1.1$^+$ cells at initial stages of infection may result in early T cell-independent IFN-γ production, which could in turn drive immunity to the predominant Th1 type of CD4$^+$ response characteristic of *T. gondii* infection (3).

The results of the present study provide a dramatic demonstration of in vivo induction of IFN-γ producing NK1.1$^+$ cells in response to microbial stimulation. In addition, our findings unequivocally establish that such cells confer strong resistance to challenge with a normally lethal pathogen. Recently, we have shown in an in vitro system that spleen cells and bone marrow derived NK cells from SCID mice produce IFN-γ when cultured with live *T. gondii* parasites or tachyzoite extract (28). This response, like that induced by the intracellular bacterium *Listeria monocytogenes* (38, 39), is dependent upon TNF-α and IL-12 released by macrophages after microbial stimulation (40, 41). Preliminary evidence indicates that the NK1.1$^+$ cells induced in vaccinated β_2m-negative mice are triggered to produce IFN-γ by a similar accessory cell dependent pathway.

In addition to demonstrating in vivo induction of protective NK1.1$^+$ cells in response to microbial infection, the results of this study provide a major example of redundancy and adaptability in the murine immune system. Furthermore, the ability of a defective host immune system to utilize alternate pathways to produce protective IFN-γ has important clinical implications. Thus, in the case of toxoplasmosis and other opportunistic infections, this property could potentially be exploited to induce T independent resistance in immunocompromised hosts.

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